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EXPLORING THE ROLE OF PDGF-D IN HEALTH AND DISEASE

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Exploring the role of PDGF-D in health and disease

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Flercelliga organismer är uppbyggda av flera sorters celler med många olika funktioner. Vissa av cellerna är specialiserade på att få hjärtat att slå, medan andra celler står beredda ifall ett sår behöver läkas, eller ser till att blod kan transporteras så att alla organismens celler kan få näring. För att organismens celler ska fungera på ett korrekt sätt behöver cellerna kommunicera med varandra. Detta sker via signalsubstanser som utsöndras av den cell som vill något. Signalen tas emot av en mottagare - en receptor, som finns på mottagarcellen. Mottagarcellen reagerar på signalen, t.ex. genom att dela sig, röra på sig eller skicka ut nya signaler. Ibland kan det bli fel i cellernas kommunikation, vilket är vanligt i olika sjukdomssammanhang. Felet kan bero på att cellerna har blivit skadade så att de inte längre kan signalera normalt, som vid cancer, då enstaka celler förlorar förmågan att svara på vissa signaler, med följden att de börjar växa på ett okontrollerat sätt.

En familj av signalsubstanser är PDGF (Platelet-Derived Growth Factor), som normalt utsöndras från bland annat blodkärlsceller. PDGF-familjen består av fyra olika typer av PDGF substanser som alla har olika funktioner i organismen, men gemensamt är att de fungerar som tillväxtfaktorer. PDGF-signaleringen är livsviktig under fosterstadiet, där specifika celler får signaler om hur de ska dela sig och vandra till platser där de behövs. Utan korrekt PDGF-signalering utvecklas därför inte organismen som den ska. En annan viktig funktion för PDGF är att se till att blodkärlen är omslutna av stödjande celler, så att de håller ihop och inte läcker. Dessutom är PDGF involverat i cancer, där för mycket PDGF-signalering kan leda till att det bildas cancerceller, eller att cancerceller stimuleras att växa.

Den här avhandlingen handlar om PDGF-D, den senast upptäckta medlemmen i PDGF-familjen, som även är den substans man vet minst om. För att försöka förstå vad PDGF-D gör har vi konstruerat en genetiskt modifierad musstam som saknar PDGF-D. I artikel I undersöker vi den musstammen och visar att PDGF-D inte är nödvändigt för normal fosterutveckling, men att avsaknaden av PDGF-D resulterar i ett lite förhöjt blodtryck. Vi visar även att PDGF-D finns i blodkärl, både i cellagret som utgör själva ”röret” och i stödjeceller som sitter runt om, samt att vissa av stödjecellerna är beroende av PDGF-D för att fungera optimalt. I Artikel II visar vi att en speciell form av cancer i bukspottkörteln som saknar PDGF-D växer mindre. Vi kom fram till att det berodde på att PDGF-D signalering påverkar en mycket liten cellpopulation, som påverkar tumörens tillväxt och utveckling. Slutligen, i Artikel III fokuserar vi på NRP1, som fungerar som en tilläggsreceptor till en annan receptor för PDGF-D och vi visar att NRP1 kan påverka PDGF-D signalering.

De forskningsfynd som presenteras i avhandlingen utvidgar kunskapen om hur PDGF-familjen fungerar och signalerar. Den ökade förståelsen för hur cellerna kommunicerar med hjälp av PDGF behövs för att kunna utveckla nya, bättre läkemedel mot sjukdomar där bland annat blodkärlen är påverkade, till exempel åderförfettning och cancer.

ABSTRACT

The platelet-derived growth factors (PDGF) and their receptors (PDGFRs) regulate growth and migration in cell populations of mesenchymal origin. The PDGF signaling system is vital for development, in neural crest formation, in organogenesis, and in maturation of the microvasculature. In the adult, PDGFs are needed to maintain homeostasis. They are also released in response to tissue injury, where they promote wound healing and neovascularization. In the adult, high expression of PDGFs is also seen in atherosclerosis, fibrosis and in malignant conditions. The PDGF family consists of four ligands that are present as dimers (PDGF-AA, PDGF-BB, PDGF-CC and PDGF-DD) and two tyrosine kinase receptors (PDGFRα and PDGFRβ). Upon ligand binding, receptor dimerization and auto-phosphorylation is induced. Downstream signaling leads to immediate effects in receptor-expressing cells, but also prolonged effects through modulation of transcription are seen.

PDGF-D is the most recently found ligand and its biological function is still unclear, although its signaling receptor PDGFRβ is mainly expressed in vascular smooth muscle cells, thus indicating a vascular role also for PDGF-D. PDGF-B, the other PDGFRβ ligand, also binds to PDGFRα, thus making PDGF-D the only ligand that signals exclusively through PDGFRβ. Moreover, PDGF-D expression is uncoupled from its signaling as it is released in a latent, full-length form requiring proteolytic cleavage for receptor binding. In contrast, the other PDGFRβ ligand, PDGF-B, is active already upon release.

PDGF signaling has been studied through a multitude of genetically modified animals, and these studies have contributed greatly to the understanding of PDGF function. In the work included in this thesis, we present the PDGF-D knockout mouse strain, and characterize the expression and function of PDGF-D *in vivo*, in both physiological conditions and in the tumor setting. We confirm that PDGF-D has a vascular expression pattern, and show that it is mainly expressed in arteries and in the endothelium, but it can also be expressed in vSMCs. We show that targeted deletion of PDGF-D affected an NG2-expressing pericyte population in the heart, and that animals lacking PDGF-D have slightly elevated blood pressure. Furthermore, we present evidence that paracrine PDGF-D signaling from the vasculature induces the production of factors from a rare PDGFRβ-expressing tumor cell subpopulation, thereby contributing to tumor growth. We also define a possible role for a co-receptor in this process. Finally, we present NRP1 as co-receptor for PDGF-D in PDGFRβ signaling, and thereby also suggest a mechanistic basis for PDGF-D-specific PDGFRβ-NRP1 complex formation and signaling. The addition of NRP1-mediated modulation adds complexity to the current model of PDGF-D/PDGFRβ signaling. Ultimately, these findings will lead to a better understanding of the role(s) of PDGF-D signaling, and thereby to improved development of tailored therapeutics for conditions where PDGF-D signaling might be dysregulated, such as atherosclerosis and cancer.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I. **Gladh H***, Folestad EB*, Muhl L, Ehnman M, Tannenberg P, Lawrence AL, Betsholtz C and Eriksson U. Mice lacking Platelet-Derived Growth Factor D display a mild vascular phenotype.
PLoS One. 2016 Mar 31;11(3):e0152276. (*Equal contribution)

II. Cortez E, **Gladh H**, Braun S, Bocci M, Cordero E, Björkström NK, Miyazaki H, Michael IP, Eriksson U, Folestad E and Pietras K. Functional malignant cell heterogeneity in pancreatic neuroendocrine tumors revealed by targeting of PDGF-DD.
Proc Natl Acad Sci U S A. 2016 Feb 16;113(7):E864-73

III. Muhl L, **Gladh H**, Folestad EB, Wang Y, Jakobsson L, Eriksson U. Neuropilin 1 is a co-receptor for Platelet-Derived Growth Factor (PDGF)-D/PDGF receptor (PDGFR)β signaling.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

CAF	Cancer-associated fibroblast
CS	Chondroitin sulfate
CSC	Cancer stem cell
CUB	Complement subcomponents c1r/c1s, Urchin EGF-like protein and Bone morphogenic protein 1
ECM	Extracellular matrix
EMT	Epithelial-to-mesenchymal transition
FACS	Fluorescence-activated cell sorting
GFD	Growth factor domain
LRP	Low-density lipo-protein receptor related protein
MMP	Matrix metalloproteinases
M_r	Relative molecular weight
mRNA	Messenger ribonucleic acid
NG2	Nerve/glial antigen 2
NRP	Neuropilin
PanNET	Pancreatic neuroendocrine tumors
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphatidylinositol 3'-kinase
SEMA3	Class-3 semaphorin
SH2	Src homology 2
TGF-β	Transforming growth factor β
TIMP	Tissue inhibitor of metalloproteinase
tPA	Tissue plasminogen activator
uPA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1 INTRODUCTION

The family of platelet-derived growth factors (PDGFs) is important during development, in adult physiology, and in a number of pathological conditions, including atherosclerosis, fibrosis, and cancer. They act by inducing survival, proliferation and migration in cells of mesenchymal origin, and are often released upon tissue injury (reviewed in (1)). Initially, the PDGF family consisted only of PDGF-A and -B, but in 2000 and 2001, two more ligands were unexpectedly discovered. These novel ligands were named PDGF-C and PDGF-D, and added new complexity to PDGF signaling, which is still not completely understood. Moreover, the biological function of the latest member in the PDGF family, PDGF-D, is obscure. The aim of the work described in this thesis was to investigate the biological role(s) and functional relevance of PDGF-D, both during physiological and pathological conditions, and thereby enhance the understanding of PDGF signaling.

This introductory chapter will mainly focus on what is known about the structure, regulation and function of the PDGFs and their receptors in physiological and pathological settings. It will also introduce NRP1 and its known interactions with PDGFs, as this thesis describes a novel role of NRP1 as a co-receptor for PDGF-D.

1.1 THE PDGF SYSTEM

The PDGF family of growth factors consists of four ligands, PDGF-A, -B, -C, and -D, and two receptors PDGFRα and PDGFRβ (2). The PDGFs were discovered already in the 1970's, and were among the first growth factors to be characterized, and the PDGF/PDGFR signaling have served as a model system for other growth factor families signaling through tyrosine kinase receptors, and is one of the most well-studied ligand-receptor systems.

1.1.1 PDGF ligands, structure and activation

The four PDGF ligands belong to the vascular endothelial growth factor (VEGF)/PDGF superfamily, which all share a conserved growth factor domain (GFD) with eight conserved cysteine residues that are important for disulphide bond formation and protein structure (3). Grouping by homology divides the PDGFs in two structural subgroups, also corresponding to the order in which the PDGFs were discovered. Thus, PDGF-A and PDGF-B form one subgroup where their N-terminal signal peptide is followed by a pro-peptide, the GFD and a C-terminal basic retention motif that allows for interactions with the extracellular matrix. PDGF-C and PDGF-D form a second subgroup as they have an additional N-terminal CUB domain (named after the first three proteins it was found in; Complement subcomponents C1r/C1s, Urchin EGF-like protein and Bone morphogenic protein 1), followed by a hinge region and the GFD, but no C-terminal retention motif (reviewed in (2)). The CUB-domains block receptor binding of the GFD, and need to be proteolytically removed before receptor binding and signaling can be induced. CUB domains are generally found in extracellular and membrane-associated proteins involved in developmentally regulated processes and are thought to mediate extracellular binding (reviewed in (4) and in (2)).

The PDGF ligands are encoded by four separate genes, which are located on different chromosomes. The *Pdgfd* gene is very large and spans over 200.000 base pairs, half of which are in the massive first intron. It is located on chromosome 11q22.3 in humans, and on 9A1 in the mouse (5-7) and is comprised of seven exons. Exon 1 encodes the signal peptide, exon 2-3 the CUB domain, exon 4 the hinge region between the two structural domains, and lastly, exon 6-7 encode the GFD (6, 8).

Biologically, the PDGFs occur as homo- or heterodimers (PDGF-AA, -AB, -BB, -CC, and -DD), which all require proteolytic processing before they can bind to their receptors. PDGF-A and -B are processed and activated prior to secretion, while PDGF-C and -D are activated extracellularly (reviewed in (2)). In PDGF-A and PDGF-B, the N-terminal propeptides are cleaved off intracellularly before secretion, by the dibasic-specific proprotein convertase furin (reviewed in (2)). In contrast to PDGF-A and PDGF-B, PDGF-C and PDGF-D are secreted in their full-length form, in which the CUB domains prevent receptor binding and needs to be cleaved off before receptor binding and signaling can occur. The extracellular cleavage is performed by serine proteases. PDGF-C is cleaved by tissue plasminogen activator (tPA), while PDGF-D is cleaved by urokinase plasminogen activator (uPA) and matriptase. However, there might be other proteases capable of cleaving PDGF-C and PDGF-D that are still unknown. The cleavage site for PDGF-D is located in the hinge region (R247/R249) between the CUB and GFD domains (9-11).

In addition to their full-length forms, PDGF-A and PDGF-B also exist in isoforms without the C-terminal retention motif; PDGF-A has an alternative splicing form, encoded by exons 1-5 and the short (3 amino acids) exon 7, while in PDGF-B, the retention motif can be removed by post-translational or extracellular processing (12-15). No additional isoforms have been reported for PDGF-C, but several splicing forms have been reported for PDGF-D. In human, the main isoform consists of 370 amino acids, and the full-length peptide chain had a calculated molecular weight of ~40.3 kDa migrates on SDS-PAGE as a M_r 90 kDa dimer, or as a M_r 55 kDa monomer (5). The cleaved, activated dimer migrated at M_r 35 kDa, and the cleaved, activated monomer at M_r 20 kDa (6). PDGF-D has a second splice variant of 364 amino acids, lacking 6 amino acids in the region upstream of the CUB-domain (5), and in mouse, a third splicing variant has been reported, which is lacking exon 6 and has no mitogenic properties in the GFD (16). Little is known about these alternate splicing forms of PDGF-D, and therefore, only the first isoform will be discussed in this work.

1.1.2 PDGF receptors, structure and signaling

PDGFs act by binding to the tyrosine kinase receptors PDGFRα and PDGFRβ, which induces downstream signaling and thereby modulates cellular functions. The PDGF receptors form dimers upon ligand binding, with different specificity for the ligands. PDGF-AA, -BB, -AB and -CC all bind to the PDGFRα homodimer and PDGF-BB and -DD bind to the PDGFRβ homodimer (reviewed in (2)). Each receptor monomer has five extracellular immunoglobulin (Ig)-like domains, whereof the three N-terminal ones are involved in ligand binding, followed by a trans-membrane domain and an intracellular split tyrosine kinase domain (reviewed in

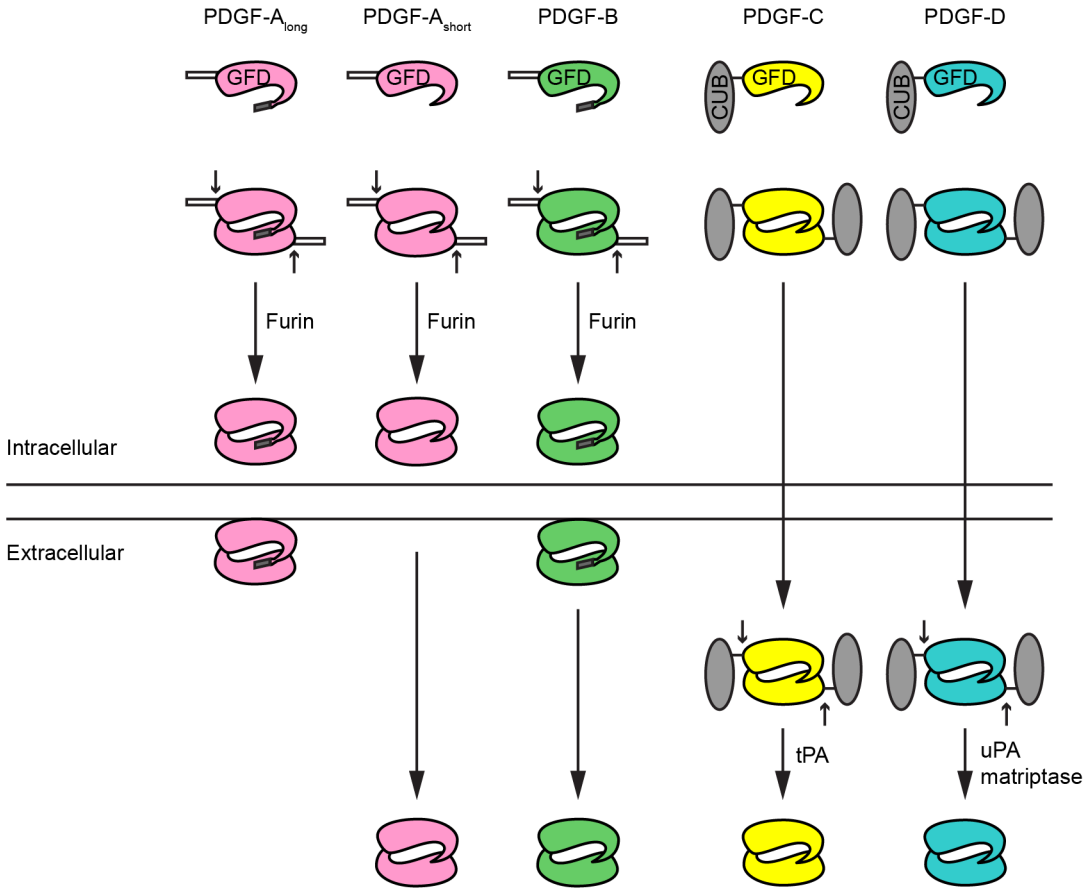

Figure 1. PDGF processing and secretion. PDGF-A and PDGF-B propeptides are cleaved off intracellularly by furin and other proprotein convertases. PDGF-A_{long} and PDGF-B are kept close to the cell of origin through ECM binding of their C-terminal retention motifs. PDGF-A_{short} is diffusible. The PDGF-B C-terminal retention motif can be cleaved off to yield a diffusible PDGF-B. PDGF-C and PDGF-D are secreted in their inactive, diffusible forms, and require proteolytical removal by serine proteases of their CUB-domains to become activated.

(1)). Binding of a PDGF ligand to a receptor monomer induces receptor dimerization, whereupon conformational changes lead to auto-phosphorylation of the intracellular tyrosine residues and enables binding of SH2 (Src Homology 2) domain signaling molecules. Structural differences between the receptor monomers affect the binding site locations and affinities for SH2, and thus also the downstream signaling.

The PDGF receptor dimers formed upon ligand binding have different specificities for the ligand dimers, and while PDGF-AA, -BB, -AB and -CC all bind to the PDGFRα homodimer PDGF-BB and -DD bind to the PDGFRβ homodimer (reviewed in (2)). All ligand dimers, except PDGF–AA, can bind to the PDGFRαβ heterodimer *in vitro*, although a biological relevance of this receptor dimer has not yet been established in physiological conditions (reviewed in (2)). However, PDGFRαβ dimers are present in certain tumors, and it is possible that also PDGF-DD signals through the heterodimeric receptor complex under pathological conditions (6, 17).

Activated PDGFRs interact with different families of SH2-domain containing molecules; some have enzymatic activity (Src, RasGAP, SHP-2, PLC-γ), others are adaptor molecules (Grb2, Nck, Shc, Crk, Alix and subdomains of phosphatidylinositol 3'-kinase (PI3K)) leading

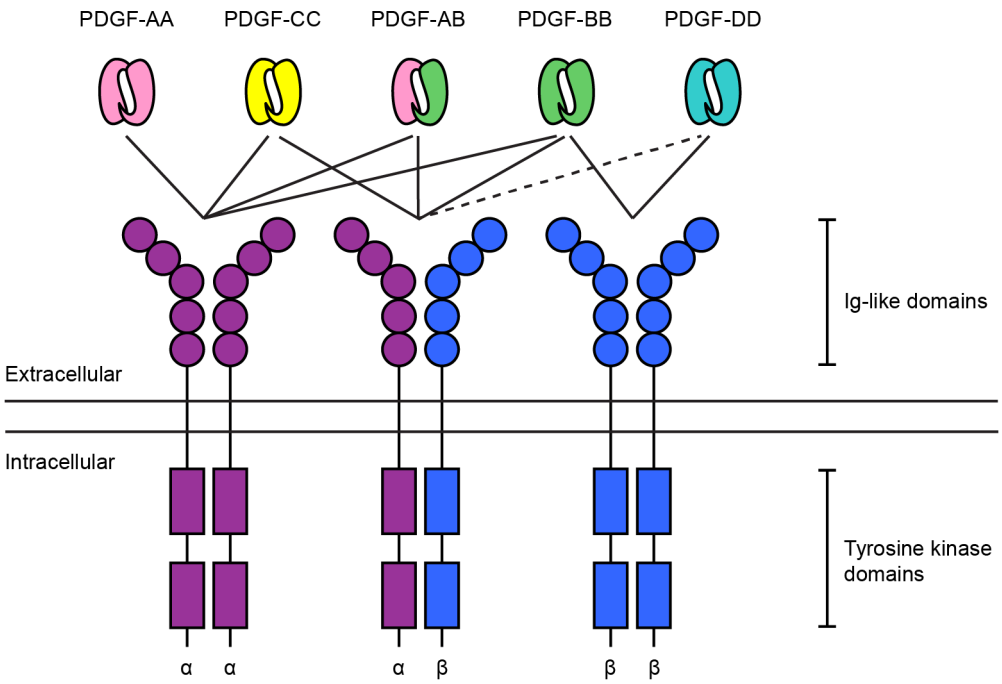

Figure 2. PDGF receptor specificity. All PDGFs except PDGF-D bind to PDGFRα, but only PDGF-B and PDGF-D bind to PDGFRβ. PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD can bind to the PDGFRαβ heterodimieric receptor, but it is still unclear whether this receptor has a physiological relevance.

to downstream signaling. PDGFRs also bind signaling enhancers (NHERF), and activate transcription factors (STAT family) (reviewed in (1) and in (18)). An important PDGFR-induced pathway is the diverse PI3K pathway, which mediates actin re-organization and migration, as well as proliferative and anti-apoptotic responses, through Akt-mediated activation of the transcription factor NF-κB. Other responses induced by PDGFRs are PLC-γ-mediated Ca^{2+} mobilization, and Ras and Src mediated activation of the Myc and Erk/MAP kinase pathways that promote proliferation (reviewed in (1)). The signaling outcome also depends on target cell-specific intracellular signaling pathways, and crosstalk between different pathways, which in some cases even counteract each other (reviewed in (1)). Most pathways are induced by all three PDGFR dimers, but the PDGFRβ intracellular domain is more potent in inducing migration, and also has unique signaling capacities needed for mural cell function (19).

Following PDGFR activation and adaptor protein binding, receptors are removed of from the cell surface by endocytosis. Vesicle forming proteins named clathrins are recruited to the PDGFR cytosolic domains to make them accumulate in clusters, which are subsequently reabsorbed into the cell and sorted for recycling or lysosomal degradation (reviewed in (20) and in (18)).

1.2 FUNCTIONAL ASPECTS OF PDGF SIGNALING

The PDGF system have been extensively studied *in vitro* and *in vivo*, and is known to be of vital importance during development, as both PDGFR null mice phenotypes are embryonic lethal. PDGFRα signaling is indispensable in organogenesis, and PDGFRβ signaling is crucial for the development of the vascular system. The PDGFRs are expressed in cells of mesenchymal origin, such as fibroblasts, smooth muscle cells and pericytes (a perivascular supportive cell type), which are often found in connective tissues, while ligands are mostly expressed in neighbouring cells. Knowledge of the sites of expression for each of the effectors is necessary when trying to understand a functional role, as this often reflects sites of biological implications. Other important parts of the regulation include proteolytic activation of the ligands, bioavailability and interactions of the ligands with the extracellular matrix (ECM).

1.2.1 Expression and regulation

PDGFRα, PDGF-A and PDGF-C are expressed already in early embryogenesis, and in greater abundance than the more vascular PDGFRβ, PDGF-B and PDGF-D during the early stages of development (reviewed in (2)). Both PDGFRs are expressed by mesenchymal cells, PDGFRα particularly by certain subtypes of progenitor cells (lung, skin, intestine and oligodendrocyte progenitors) while PDGFRβ is strongly expressed in vascular smooth muscle cells (vSMCs) and pericytes throughout vascular system (reviewed in (2)). PDGFRβ is also often used as a pericyte marker in adult tissue (23). It is noteworthy that no physiological function for the PDGFRαβ heterodimer has been identified, and that PDGF and PDGFR expression levels are lower in adult (reviewed in (24)). The detailed expression of the ligands is complex, but in general, PDGF-B is expressed by endothelial cells, megakaryocytes and neurons while PDGF-A and PDGF-C are expressed in epithelial cells, muscle, and neuronal progenitors with a partial overlapping expression pattern. Their expression patterns have been reviewed in detail (2).

The expression pattern of PDGF-D, the most recently found PDGF family member, is not as well characterized as for the other PDGFs. However, *Pdgfd* messenger ribonucleic acid (mRNA) is known to be present in most adult tissues in both human, rat and mouse (5, 6, 25) further discussed in Paper I). The cellular sources of PDGF-D are not very well described, probably as a consequence of lack of both good commercial antibodies and in situ hybridization probes against human and especially mouse PDGF-D. Moreover, until our recent publication of the *Pdgfd*$^{-/-}$ mouse strain with a *LacZ* reporter gene expressed under the *Pdgfd* promoter, reporter mice have also been lacking (see Paper I (26)). In spite of the lack of good commercial tools for histological detection of PDGF-D, there are a number of reports of PDGF-D expression. These observations are summarized below, in relation the expression of PDGFRβ and PDGF-B.

Similar to PDGF-B and PDGFRβ, PDGF-D is expressed at low levels in the normal adult vasculature, although there have been some discrepancies regarding its exact localization.

PDGF-D expression has been reported in all three vascular layers (endothelium, tunica media, tunica adventitia), by endothelial cells, vSMCs and fibroblasts (8, 27, 28). During development, PDGF-D and PDGFRβ are co-expressed in arterial vSMCs, and both are present in blood vessels lining the vertebra, as well as in skin, skeletal muscle, liver, and lung (29). The same study also reported PDGF-D mid-gestational expression in epicardial and endocardial cardiomyocytes, and in myocardium, whereas cardiac PDGFRβ was mainly observed in the vasculature (29). In the developing avian heart, PDGFRβ expression has been reported also in the atrio-ventricular cushions that will develop into cardiac valves and connect to the septa, while PDGF-B was seen in endothelial cells, vSMCs, nerves and in the developing ventricular septum (30).

In the adult heart, PDGF-B and PDGFRβ levels are very low, but PDGFRβ is present in vSMCs (reviewed in (24)). Presence of PDGF-D has been reported in adult myocardium (29). In the kidney, PDGFRβ is expressed by glomerular mesangial cells and vSMCs, while PDGF-B is expressed by endothelial cells. In developing and adult kidney, PDGF-D expression is present in multiple cell types of mesenchymal origin, such as arterial vSMCs and fibroblasts, both in mouse and human. PDGF-D is also expressed in neighbouring cells, such as glomerular podocytes and other epithelial cells of the nephron (5, 31, 32). The glomerulus was recently reported to display a species-specific expression pattern; in human, PDGF-D expression was seen in podocytes but not in mesangial cells (specialized glomerular pericytes), whereas the opposite was seen in the mouse (32). In the anterior part of the eye surrounding the lens, PDGF-D expression has been reported in the epithelial layers of the ciliary body and iris, both during development and in adult tissue. PDGFRβ was expressed in the epithelium of the lens and cornea and in the mesenchyme surrounding the optic cup during development (33, 34). There are also reports on hematopoietic stem cells expressing PDGF-D, and adipose-derived stem cells co-expressing PDGF-D and PDGFRβ but not PDGF-B (35, 36). PDGF-D expression has also been observed in the rat central nervous system, both during development (E16) and in adult tissue (37).

In summary, PDGF-D is present in both developing and adult tissues, where it is expressed both in cells of mesenchymal origin (vSMCs, mesangial cells and fibroblasts) and in neighboring cells, such as endothelium and certain epithelial tissues. This expression pattern suggests that PDGF-D can act through both paracrine and autocrine modes of signaling. Recently, we published a report on a *Pdgfd*$^{-/-}$ mouse strain, carrying a *LacZ* reporter gene inserted into the PDGF-D gene to characterize the global and vascular expression of PDGF-D in detail (26). Our findings are further discussed in Paper I.

Regulation and modulation of PDGF signaling

As discussed above, the downstream signaling pathways appear to be similar between the two PDGFRs, while the expression of both receptors and ligands appear to be dependent on spatial and temporal regulation together with tissue specific environmental factors.

During physiological conditions, PDGFs mainly act by paracrine signaling, and consequently, the bioavailability of the ligands and receptors in different tissues is a major regulatory aspect, which is in turn depending on a multitude of regulatory mechanisms. A major regulatory factor for PDGF-C and PDGF-D is proteolytic activation that is needed to enable receptor binding (discussed in section 1.1.1). The proteolysis is regulated through local expression and activation by tPA, uPA and matriptase, and possibly also by other unknown proteases (9, 11, 38). Interactions with extracellular elements serve to prevent diffusion and/or receptor activation, and also mediate establishment of signaling gradients that are important for chemotaxis (reviewed in (39)). The retention motif bearing isoforms of PDGF-A and PDGF-B bind to heparan sulfate, and in blood, PDGF-B availability is also regulated through binding of the plasma protein α_2-macroglobulin. It is not clear to what extent the latently secreted PDGF-C and PDGF-D interact with ECM through their CUB-domains (reviewed in (2)). However, unpublished observations from our lab indicate that full-length PDGF-D is more diffusible than the cleaved, activated growth factor. Signaling modulation through crosstalk with other signaling pathways has been described between PDGFRβ and the adhesion receptor integrin $\alpha_v\beta_3$ (reviewed in (1)), thus indicating interference with or from cell-cell or cell-ECM contact. Modulation by co-receptors can act to block, enhance or alter intracellular signaling pathways or affect receptor availability. For PDGFs, this type of interactions have been reported together with low-density lipoprotein receptor related protein (LRP) (40), the uPA receptor (uPAR) (41) and Neuropilin 1 (NRP1) (42, 43). The role of NRP1 in modulation of PDGFRβ signaling will be further discussed below and in Paper III. Receptor availability can be modulated through receptor trafficking upon endocytosis. For PDGFRβ, endocytosis has been reported to be modulated by LRP (44) and Eph/ephrin signaling (45). Moreover, the metalloproteinase ADAM10 has been identified as a sheddase that cleaves off the extracellular domain for PDGFRβ, although a functional role of PDGFRβ shedding has not yet been found (46). Receptor autoactivation upon increased levels of reactive oxygen species (ROS) can induce PDGFRβ-mediated proliferation and migration (47, 48).

1.2.2 Physiological roles

A substantial part of the knowledge of the basic PDGF functions are derived from *in vitro* studies, in which the PDGFs have been shown to mediate survival, proliferation and migration. There have also been extensive studies of gene expression, using transgenic mouse models and different loss- or gain-of-function strategies providing a deeper understanding of many complex physiological functions *in vivo*. During embryonic stages, PDGFs act to guide proliferation, recruitment and migration of cells, while in adult tissues, they mainly act to maintain homeostasis. In this section, the roles of PDGFs in development and adult tissues are discussed, with focus on the vascular function, as this is the main site of expression for PDGFRβ, the signaling receptor of PDGF-D

PDGFs during development

Although *in vitro* studies have shown that the downstream signaling pathways between PDGFRα and PDGFRβ largely overlaps, ablation of the two receptors give rise to very different phenotypes (reviewed in (49)).

PDGFRα and PDGF-A ablation gives rise to similar, multifaceted, phenotypes, with defects in oligodendrocytes, chondrocytes, neural crest, alveolar SMCs, kidney fibroblasts, chorioallantoic plate, Leydig cells, intestinal mesenchyme and dermis. *Pdgfa*$^{-/-}$ animals die perinatally due to respiratory problems, while *Pdgfra*$^{-/-}$ embryos die already at mid gestation (reviewed in (49)). The PDGF-C phenotype is background dependent; *Pdgfc*$^{-/-}$ mice on a 129S1/Sv genetic background die perinatally from feeding and respiratory problems due to a cleft palate, whereas *Pdgfc*$^{-/-}$ mice on a C57BL/6 genetic background survive, but display cerebral ventricular malformations, abnormal vascularization and skeletal deformations (50, 51). Loss of both PDGF-A and PDGF-C phenocopies the developmental defects in *Pdgfra*$^{-/-}$ mice (50).

The *Pdgfrb*$^{-/-}$ and *Pdgfb*$^{-/-}$ phenotypes both gives rise to a general loss of vSMCs, and embryos die due to internal bleeding around birth with a slightly worse phenotype in *Pdgfrb*$^{-/-}$ animals. Both *Pdgfrb*$^{-/-}$ and *Pdgfb*$^{-/-}$ animals also display abnormal cardiac innervation (52, 53). Moreover, both PDGFRα and PDGFRβ are needed for cardiac neural crest development (reviewed in (2)).

Given the similar phenotypes of the *Pdgfrb*$^{-/-}$ and *Pdgfb*$^{-/-}$ mice, PDGF-D knockout mice were not expected to have a severe phenotype, at least not during embryonic development. However, there are some slight deviations between the *Pdgfrb*$^{-/-}$ and *Pdgfb*$^{-/-}$ phenotypes; PDGFRβ-positive pericyte progenitors are present in skeletal muscle, skin and the adrenal gland in the less severe *Pdgfb*$^{-/-}$ mouse phenotype (reviewed in (1)). It has been suggested that compensational PDGF-D signaling could be responsible for these phenotypic differences.

Little is known about the developmental role of PDGF-D; expression data indicate the presence of PDGF-D in cardiac, renal and cerebellar tissues, and a role in lens formation has been suggested (29, 31, 33, 37). Nevertheless, ligands need receptors to exert their functions, and with PDGFRβ as the only known receptor for PDGF-D, their functional roles are expected to be coherent. PDGFRβ is mainly expressed in vSMCs/pericytes and thus, PDGF-D is also expected to have a functional role for these cells.

The role of PDGFRβ in angiogenesis and vascular maturation has been extensively studied using different mouse models and reporter genes, leading to the understanding that PDGF-B signaling through PDGFRβ is crucial for proper pericyte recruitment and attachment to the vasculature (54). During angiogenesis, the endothelium adopts a more active phenotype to build new vessels. PDGF-B is secreted from endothelial *tip cells* at the angiogenic front to attract PDGFRβ-expressing pericytes or mural cell progenitors that migrate along the newly formed vessel sprouts (54). The tip cells are VEGF-sensitive and instruct neighboring

endothelial cells to take on the proliferative *stalk cell* phenotype through Notch signaling, and thereby promote to elongation of the vascular sprouts (55). In the newly formed endothelium, PDGF-B signaling through PDGFRβ promotes maturation and stabilization. Moreover, their signaling also controls mural cell fate and proliferation (56, 57). Consistently, PDGF-B expression is strong also in growing arteries, where mural cells are actively recruited (23, 54). Later on, and in the adult stage most endothelial cells display a third, resting *phalanx cell* phenotype (58). Here, stabilizing pericyte-to-endothelial cell signals regulate the endothelial proliferation, survival, migration and differentiation, thereby repressing unwanted angiogenesis. Vessels that lack pericytes are leaky and have increased capillary diameter. The endothelial cells in such vessels display hyperplasia and excessive folding of the luminal membrane, accompanied by altered expression of junction proteins (59). Thus, pericytes act as negative regulators of endothelial proliferation, and also instructs the endothelium to keep a smooth luminal surface membrane (reviewed in (2)).

As described in section 1.2.1, many stem and progenitor cells also express PDGFs during development, and their activity is often regulated by PDGF signaling from neighbouring cells. One example is in the Sertoli cells of the testis, where both PDGF-B and PDGF-D are expressed and regulate proliferation and migration of spermatogonial cell precursors (60).

Physiological role in adult

The embryonic lethality of *Pdgfra*$^{-/-}$ and *Pdgfrb*$^{-/-}$ animals has lead to a focus on the developmental functions of these receptors. The PDGFRs are expressed in lower levels also in adult, dormant tissues (reviewed in (24)), and it has been suggested that the role of PDGF signaling is less important in the adult physiology than in the developmental. Consistently, pharmacological blocking of PDGFR signaling by the tyrosine kinase inhibitor Imatinib (also targeting c-kit and Abl) is tolerated relatively well (61). Imatinib treatment did, however, give adverse effects from the gastrointestinal system, accompanied by myalgia and edema, indicating sites of PDGFR activation in the adult (61). Indeed, PDGFRα is expressed in the gut, and PDGFRβ-expressing perivascular cells control vessel integrity and interstitial fluid pressure (62). Interestingly, a PDGF-D neutralizing monoclonal antibody (CR002) has been evaluated in a Phase I trial. Antibody-mediated amelioration of proteins produces few side effects, and is as close to a knockout that one can get in the human situation (63).

Nevertheless, PDGFs are needed in adult tissue homeostasis, and mouse models of partial, transgenic and conditional loss or gain of function have contributed to the understanding of PDGF signaling in adult settings. One important model is the *Pdgfb*$^{ret/ret}$ mouse strain that lacks the PDGF-B retention motif, which normally retains the secreted ligand close to the endothelial cell, as a chemotactic signal for vSMCs. The *Pdgfb*$^{ret/ret}$ mice survives until adult age, but are smaller than its wildtype littermates, and suffers from a vascular phenotype leading to renal and retinal dysfunctions. This model has also shown that the PDGF-B retention motif is necessary for proper pericyte recruitment and attachment to the capillaries (64). Further studies of the *Pdgfb*$^{ret/ret}$ mouse strain has also lead to the understanding that

pericytes are needed to maintain the blood brain barrier, and that this is regulated through PDGF signaling (65).

As implied by the name, PDGFs were originally isolated from platelets, where they play an important role in wound healing. Platelets store PDGF-A, -B, and -C and other growth and clotting factors in α-granules, which are released upon tissue damage, in order to prevent blood loss and maintain homeostasis (66, 67). PDGF signaling is needed throughout the wound healing process, and contributes to a number of different events including inflammation, formation of granulation tissue, re-epithelialization, remodeling and neovascularization ((66) and reviewed in (1)). PDGF-B has been shown to be especially potent and is released from platelets immediately upon wounding, and stimulates the healing process through increased fibroblast proliferation, ECM production and neovessel formation (reviewed in (2)). Recombinant human PDGF-B (Becaplermin) is used in the clinic to enhance wound healing (68). In similarity to PDGF-B, PDGF-D also serves in recruitment and proliferation of monocytes/macrophages and vSMCs (69) and stimulates secretion of ECM, as well as matrix degradation factors (MMPs/TIMPs) by vSMCs and fibroblasts (70). Part of the wound healing process is also neovessel formation, where PDGFs induce secretion of VEGFA to promote angiogenesis, which is also guided through hypoxia and ECM scaffolding (reviewed in (71)). Other cases when adult physiological angiogenesis is needed include the adaptive response of cardiac and skeletal muscle upon physical exercise, and in the uterine cycle, where the tissue is shed and rebuilt in a cyclic manner.

Another function of PDGFRβ-expressing mural cells is in control of interstitial tissue pressure, through maintaining vascular impermeability and contraction of ECM, which modulated by both PDGF-B and PDGF-D signaling (69, 72).

PDGFs regulate mesenchymal stem cell functions also in adult tissues. During the uterine cycle, PDGFRβ-expressing cells found in perivascular locations have been suggested to be responsible not only for angiogenesis but also for the cyclical growth of the endometrium (73). A special function in certain stem cells is also seen for PDGF-D. In contrast to PDGF-B, PDGF-D is occasionally co-expressed with PDGFRβ, thus facilitating self-maintaining autocrine signaling loops that promote proliferation and migration, as seen in adipose tissue derived stem cells (74).

In summary, as a PDGFRβ ligand, PDGF-D may induce responses that overlap with those of PDGF-B, such as survival, proliferation, migration, vascular maturation, and regulation of interstitial fluid pressure and ECM homeostasis. Thus, there may be some functional redundancy in PDGFRβ signaling between PDGF-D and PDGF-B. In consistence, we recently reported our finding that the *Pdgfd*$^{-/-}$ mice display no major phenotype and survive to adulthood. However we also reported that PDGF-D appear to be involved in mural cell maintenance during physiological conditions, which is further discussed in Paper I (26).

1.3 PATHOLOGICAL ASPECTS OF PDGF SIGNALING

Dysregulation of PDGF activity is seen in a number of common pathological conditions, such as fibrosis, atherosclerosis and cancer. To better understand the role of PDGF signaling in these conditions, extensive studies have been performed, including different overexpression strategies *in vivo*. These studies indicate that PDGFs have strong impact on the progression of these pathologies, through promotion of excessive proliferation, ECM synthesis and migratory signals, commonly affecting different types of SMCs and/or fibroblasts.

1.3.1 Fibrosis

Fibrosis is a common condition that is mechanistically similar to exaggerated wound healing, but also occurs in response other types of tissue damage. The pathological process is characterized by excessive deposition of collagens and other ECM components in the interstitium, leading to scarring and disruption of tissue function. These events are also firmly connected to transforming growth factor β (TGF-β) signaling, which is a very potent inducer of ECM secretion (75). Thus, a central player in fibrosis is the fibroblast, a heterogeneous cell type that varies between organs, which responds to TGF-β, PDGF, and other stimuli (reviewed in (76) and in (2)).

PDGFs contribute to the fibrotic process by proliferative and chemotactic signaling, especially through myofibroblast expansion (reviewed in (77) and in (2)). All four PDGFs are upregulated during fibrosis, and cause massive fibrotic responses when overexpressed in animal models (reviewed in (2)). High PDGF expression has been described in pulmonary, hepatic, renal and cardiac fibrosis, although the expression and regulation of each PDGF ligand appears to be organ-specific also in fibrosis (reviewed in (77)). Interestingly, crosstalk has been reported between PDGFs and the pro-fibrotic TGF-β, which seems to contribute to the spatial regulation of the different ligands. In the case of PDGF-D, TGF-β has been shown to promote signaling in cardiac fibroblasts, but inhibits PDGF-D expression in the lung (78, 79). The pro-fibrotic potential of PDGF-D in heart and kidney has been demonstrated by overexpression studies *in vivo* (29, 32, 80), and PDGF-D/PDGFRβ signaling is also implicated in liver fibrosis (81-83). PDGFRβ-mediated fibrotic responses are executed by fibroblasts, myofibroblasts and specialized pericytes, such as hepatic stellate cells and renal mesangial cells that have been activated into a fibroblast-like state. Like other pericytes, they express PDGFRβ (84), and PDGF-D has been shown to exhibit mitogenic and fibrogenic effects on these cells (81, 83, 85, 86). Similar to the other PDGFs, PDGF-D contributes to accelerated ECM deposition. In both hepatic stellate cells and cardiac myofibroblasts, PDGF-D has been shown to upregulate tissue inhibitor of metalloproteinase (TIMP)-1, which attenuates matrix metalloproteinase (MMP) activity, thereby decreasing ECM degradation (78, 85). Notably, PDGF-D-mediated ECM remodeling is implicated in epithelial-to-mesenchymal transition (EMT) in cancer (87), a process that is activated also in fibrosis and chronic inflammation, (reviewed in (88)). Thus, it is not unlikely that PDGF-D contributes to EMT also in fibrosis, but further studies are needed on this subject.

In severe fibrosis, the irreversible tissue scarring leads to loss of tissue function, and therefore early anti-fibrotic/anti-inflammatory treatments are used. Several studies suggest PDGF-D as a suitable therapeutic target in kidney fibrosis, and an anti-PDGF-D monoclonal antibody (CR002) has been shown to prevent renal fibrosis in mice (82, 86, 89). Also, the *Pdgfd*$^{-/-}$ mice (presented in Paper I) show reduced renal fibrosis upon experimental induction of renal scarring (32).

1.3.2 Atherosclerosis and other vascular pathologies

Cardiovascular disease is a major cause of death in the western world. A major risk factor is atherosclerosis, a process in which lipid-containing plaques are slowly formed in the vascular wall. With time, the plaques may rupture and cause thrombosis or internal bleedings that can be life-threatening. Atherosclerotic plaques appear in vessel areas exposed to low shear stress, turbulent flow and oscillating flow (reviewed in (24)). Plaques are formed over decades, initially through lipid accumulation, followed by endothelial dysfunction and infiltration by macrophages and other immune cells into the vascular wall, leading to chronic inflammation.

In contrast to the healthy vasculature that expresses only low, or undetectable levels, of PDGFs, all four PDGF ligands are present in the atherosclerotic vessel wall, and are mainly secreted by endothelial cells, vSMCs and macrophages. Also, both PDGF receptors are upregulated, and expressed by vSMCs and macrophages ((28, 70) and reviewed in (24)). PDGFRβ expression is stronger than that of PDGFRα, and PDGFRβ signaling has been shown to be a driving force in the atherogenic process through chemokine signaling that induces leukocyte migration (90). In the later stages, active vSMCs play a central role, as they proliferate and contribute to the thickening of the vascular wall (reviewed in (91)). Atherogenic stimuli (ECM, cytokines, shear stress, reactive oxygen species and lipids) promote the switch from a contractile to synthetic phenotype of vSMCs, which is also associated with increased PDGFRβ expression and signaling (reviewed in (92)). PDGF-B and PDGF-D are both upregulated in endothelial cells exposed to atheroprone blood flow, and promote the vSMC phenotypic switch ((93) and reviewed in (24)). For PDGF-D, this is mediated through upregulation of the differentiation repressor gene Kruppel-like factor-4, and downregulation of contractile proteins, such as α-smooth muscle actin (αSMA) and the smooth muscle myosin heavy chain (93). PDGFRβ, PDGF-B and PDGF-D also are induced during monocyte-to-macrophage differentiation, and they are all strongly expressed in fatty streaks, where PDGFRβ signaling has a strong impact on monocyte/macrophage migration (28, 70). Moreover, PDGFs promote stabilization through ECM synthesis, as well as ECM remodeling through MMP and TIMP expression (27, 70).

Notably, recent studies have reported genetic variations in the form of single-nucleotide polymorphisms (SNPs) in the *PDGFD* gene to be associated with coronary artery disease (94), and non-hypertensive intra-cerebral hemorrhage (95). This indicates that aberrant PDGF-D signaling could be causative in atherogenesis and cardiovascular disease, emphasizing the importance of PDGF-D in these conditions.

PDGFs in response to vascular injury, rejection and restenosis

Vascular injury, restenosis and chronic rejection gives rise to conditions that resembles atherosclerosis. Upon vessel injury, medial vSMCs migrate from to the intima, where they proliferate and switch to a synthetic phenotype in a PDGFRβ dependent manner, resulting in neointimal hyperplasia. PDGF-D is upregulated in the intima following angioplasty, and is also thought to contribute in this process, next to PDGF-B ((27) and reviewed in (24)). The response to chronic cardiac allograft rejection also manifests as a vascular disease, and is histologically characterized by concentric luminal stenosis (vascular narrowing) (reviewed in (96)). In rat cardiac allografts, PDGF-D, but not PDGF-B, was induced, and increased the pro-fibrotic and pro-arteriosclerotic responses leading to chronic rejection, through the TGF-β1 pathway (97).

PDGF signaling is of importance also in vascular pathologies involving the blood brain barrier integrity in the central nervous system. In stroke, PDGF-C signaling through PDGFRα is involved in the acute opening the blood-brain barrier (98, 99). In the chronic disease named amyotrophic lateral sclerosis (ALS), high neuronal expression of PDGF-C leads to disruption of the blood-spinal cord barrier, and contributes to the early onset of the disease (100).

1.3.3 Cancer

In cancer, malignant cells grow in an uncontrolled manner and disrupt normal tissue functions. The six hallmarks of cancer established by Hanahan and Weinberg include proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (101). The tumor is built up of cancer cells, some of which have stem cell properties, and others have invasive capabilities. The tumor microenvironment also contains stromal cells, which include cancer-associated fibroblasts (CAFs), immune inflammatory cells, endothelial cells and pericytes as well as local and bone marrow-derived stromal stem and progenitor cells (reviewed in (102).

The first connections between PDGFs and cancer arose in 1983, when PDGF-B was sequenced and proved to be similar to v-sis, the transforming protein of simian sarcoma virus (103, 104). Today, all PDGFs and PDGFRs have been connected to different tumor types, and are known to be capable drivers of tumorigenicity through several of the cancer hallmark functions; proliferation, angiogenesis and migration (reviewed in (18)).

PDGF ligand overexpression is seen in carcinomas, tumors of endodermal or ectodermal origin, where they promote tumorigenesis and metastasis through recruitment and stimulation of stromal mesenchymal cells in a paracrine manner. PDGFs can act through autocrine signaling loops in cases where the tumor cells express the receptor (reviewed in (105)). One example of autocrine signaling is in dermatofibratoma protuberans, where PDGF-B is expressed under a collagen promoter, resulting in a COL1A1/PDGF-B fusion protein from which active PDGF-B is cleaved off (106).

Constitutionally active receptors are seen in tumors derived from PDGFR-expressing mesenchymal cell populations, such as sarcomas and glioblastomas, where they drive proliferation and clonal expansion through autocrine signaling (reviewed in (1)). These receptors are the result of gene translocations leading to fusion proteins as seen in hypereosinophilic syndrome, caused by a FiP1L1-PDGFRα fusion (107), and chronic myelomonocytic leukemia, caused by an ETV6-PDGFRβ fusion (108).

Other types of genetic aberrations in PDGFs and PDGFRs have also been observed to cause cancer in the case of PDGF signaling. For PDGFRα, activating point mutations are commonly seen in gastrointestinal tumors (GIST) (109), as are gene duplications in glioblastomas (110). Overexpression of receptors can also lead to auto-activation (reviewed in (111)).

PDGFRβ signaling in the tumor

In solid tumors, PDGFRβ expression is mainly confined to stromal cells, such as CAFs, vSMCs and pericytes (reviewed in (112)), where PDGFRβ is important in vascular stabilization. Tumor vessels are often leaky and fragile due to lack of supporting pericytes (reviewed in (102)). Both PDGF-B and PDGF-D, signaling through PDGFRβ, stabilizes the vasculature through recruitment of pericytes (113), thereby enhancing vascular function and contributing to tumor growth. Pericyte PDGFRβ signaling also provides survival signals to the endothelium, for example by upregulating the anti-apoptotic gene *Bcl2l2* (114). PDGFRβ expression in CAFs is associated with aggressiveness and metastasis, and PDGFRβ signaling in both CAFs and pericytes promote high interstitial fluid pressure in tumors through ECM interactions (115).

PDGF-D signaling through PDGFRβ in cancer

PDGF-D has been reported to be a driver in tumorigenesis in different cancers, such as prostate and renal cell carcinoma, pancreatic adenocarcinoma, glioblastoma, schwannoma (derived from peripheral glia cells) and melanomas (reviewed in (116)). A major difference between the PDGFRβ ligands, PDGF-B and PDGF-D, is that PDGF-D requires proteolytic activation, and thus, the enzymes that activate PDGF-D, uPA and matriptase, needs to be present in the microenvironment, as has been shown in prostate cancer (9, 11). The role of uPA in tumor growth, angiogenesis and metastasis is well studied (reviewed in (117)). For matriptase, a feedback loop has been reported, where the activated PDGF-D upregulates matriptase (118). Once activated, PDGF-D promotes proliferation, and stimulation of tumor cell growth (119). Similar to physiological conditions, PDGF-D and PDGF-B interact with the stroma through recruitment of PDGFRβ-expressing cells (vSMCs and macrophages), and expression of VEGF and MMPs (120-124).

Coherently, gene expression profiling has shown that PDGF-D associates with genes active in cell adhesion, wounding, and immune system processes (17). Moreover, in dermis, non-malignant PDGF-D overexpression has been shown to increase interstitial fluid pressure (69). Both vascular stability (or impermeability) and high interstitial pressure act to impair drug

delivery, as the drug is maintained in the blood stream. Targeting of PDGFRβ signaling improves drug delivery and efficacy of chemotherapy (125, 126). Inhibition of PDGF-D has been shown to attenuate growth, invasion and angiogenesis in a xenograft model of gastric cancer (127). However, in breast cancer PDGF-D blockade also promoted lymphatic metastasis by activation of CXCR4 (128).

PDGF-D in EMT, invasion and metastasis

PDGF-D and PDGFRβ has also been implicated in invasion and metastasis, through the process of EMT, in which epithelial cells lose their apical-basal polarization and cell-cell adhesion to acquire a migratory, fibroblast-like phenotype ((129, 130) reviewed in (131)). During physiological conditions, embryonic EMT is essential for many developmental processes, and in adult tissue it is implicated in wound healing (reviewed in (131)) and in the pathological process of fibrosis (reviewed in (88)). In cancer, tumor cells reactivate the processes of EMT to become invasive and metastatic which is associated with upregulation of mesenchymal markers involved in invasion and migration (reviewed in (132)). PDGF-D appears to regulate the process of EMT on several levels, partially through the NF-κB and Notch pathways (87, 121). This leads to downregulation of the epithelial adhesion protein E-cadherin, which in prostate cancer takes place through inhibition of microRNA-mediated regulation of the transcription factors Snail, Twist and ZEB1 (133-136). PDGF-D has also been shown to upregulate expression of MMPs and the cytoskeletal protein vimentin and other mesenchymal markers (124, 137), to promote degradation of basement membranes and ECM and thus also angiogenesis, metastasis and invasion.

PDGF-D and cancer stemness

There have been different theories on how tumors grow. Today, one that is favored by many researchers is the cancer stem cell (CSC) hypothesis. CSCs constitute a small subset of cells with the ability form new tumors when implanted into SCID mice, where they both self-renew and differentiate into more mature phenotypes. These cells only make up a small part of the total bulk of cancer cells, but are the main target to treat malignant and metastasizing disease (reviewed in (116) and in (102)). The origin of the CSCs is not clear, but they may be derived from adult stem cells, or more differentiated progenitor cells, that are present in normal tissues, and the source may also differ between tumors (reviewed in (102)). The traits of CSC and EMT-transdifferentiated cells overlap to a large extent, and therefore, PDGF-D signaling through PDGFRβ has been suggested to be involved also in cancer stemness. It has been shown that PDGF-D transformed NIH-3T3 fibroblasts show CSC features, such as anchorage-independent growth in soft agar and ability to induce tumors in nude mice (119). Moreover, several studies have reported that high expression of PDGF-D induces EMT, and SCS-like capabilities in cancer cells, thus contributing to tumor aggressiveness (136, 138).

Recently, we published a study showing that in a RIP1-TAg2 model of neuroendocrine pancreatic cancer, the tumours in *Pdgfd*$^{-/-}$ mice developed more slowly than those in *Pdgfd*$^{+/+}$

littermates. The possibility that this delay is because of altered CSC capacity is further discussed in Paper II.

1.4 NEUROPILIN 1

Neuropilin 1 (NRP1) and NRP2 are multifunctional trans-membrane co-receptors for members of the class-3 semaphorins (SEMA3) in neuronal axon guidance and vascular endothelial growth factors (VEGFs) in angiogenesis. However, interactions of NRP1 with ligands from other signaling systems, such as TGF-β1, hepatocyte growth factor (HGF) and fibroblast growth factor (FGF) have been reported (reviewed in (139)).

There is a growing body of evidence showing that PDGFR signaling can be modified by NRP1 (42, 43, 140-144), and in Paper III, we provide evidence that NRP1 can act as a co-receptor for PDGF-D. In this section, NRP1 and its functions as a co-receptor for the structurally related members of the PDGF/VEGF superfamily are described.

1.4.1 Structure, function and expression of NRP1

The NRPs consists of seven sub-domains where the first five are extracellular; two CUB domains (a1 and a2), two coagulation factor V/VIII domains (b1 and b2) and a meprin, A5 μ-phosphatase domain (c). Following the trans-membrane domain, NRPs contain a short cytosolic tail with a PDZ (Post synaptic density protein, Drosophila disc large tumor suppressor, and Zonula occludens-1 protein) binding domain (reviewed in (145)). Full-length NRP1 protein is present as a 130 kDa species, but there are also several splice variants that are less studied, and will not be discussed in this thesis (reviewed in (146)).

Ligand binding occurs at different sites of NRP1; SEMA3A binding requires the first three NRP1 sub-domains; a1, a2, and b1, whereas VEGF-A binds primarily to a negatively charged cleft in the b1 domain but also to the b2 domain (147, 148). VEGF-A_{165} binds to NRP1 through a [KPRR] motif in the C-terminus, where the last arginine is crucial for binding (148, 149). The C-terminal arginine is shared by most of the NRP1-binding VEGF isoforms (reviewed in (146)). It has been suggested that NRP1 is present as homo- or heterodimers, but it is also possible that they oligomerize to form high-affinity binding sites for ligands (150).

NRP1 is expressed by a variety of cells, including vSMCs (143), endothelial cells, neurons, and different epithelial cells (reviewed in (151)). In the vasculature, NRP1 is mainly expressed by arterial endothelial cells, and during angiogenesis also by neural progenitors and macrophages. NRPs are also commonly expressed by tumor cells and tumor vasculature (reviewed in (152)). Studies of knockout and transgenic mouse models have shown that NRP1 is crucial for neuronal, cardiac and vascular development, and *Nrp1*$^{-/-}$ animals die at E10-12.5, with VEGF-A/NRP1 signaling-dependent defects in angiogenesis, vascular branching and topographic origin of the coronary arteries (153-155). It is also clear that NRP1 expression requires tight regulation; when overexpressed, NRP1 resulted in a lethal (E12.5) phenotype with excessive capillary growth, hemorrhages, cardiac malformation and neuronal defects (156). Also, the cytoplasmic part of NRP1 is not needed for survival, as mice expressing a truncated NRP1 protein displayed a mild phenotype with increased retinal arteriovenous crossings, and reduced number of arteries in heart, kidney and skeletal muscle (157, 158).

In conclusion, the NRP1 co-receptor has a vital role in arteriogenesis and angiogenesis, and it is expressed in PDGFRβ-expressing vSMCs and neighbouring endothelial cells. Thus, the correlating spatio-temporal expression of NRP1, PDGFRβ and the PDGFRβ ligands would facilitate their interaction.

1.4.2 NRP1-mediated modulation of signaling

Neuropilins are mainly modulators of signaling (reviewed in (146)), and appear to induce situation-specific responses. Enhancing functions are generally seen for SEMA3, VEGF-A, and PDGF ligands, while NRP1 has an inhibitory function for TGF-β. Interestingly, in angiogenesis, NRP1 enhances the endothelial tip cell phenotype through VEGFR2 signaling while it inhibits the stalk cell phenotype through inhibition of TGF-βR1/2, thus driving two different responses in the same cell type (reviewed in (159)). How NRPs exert their functions is still unclear, but suggested mechanisms include ligand capture, receptor inhibition, and competitive binding between ligands (160-162). The intracellular alteration of responses is mediated through enhanced receptor trafficking or enhanced cell migration or adhesion through direct or indirect interaction with integrins (141, 143, 163, 164).

Signaling occurs in both *cis* and *trans*. So far, signaling in *trans* has mainly been reported in tumor cells, where it counteracts angiogenesis by interference with VEGFR2 trafficking. NRP1 signaling in *trans* can also mediate adhesive functions, and thereby modulate the signaling time frame, or enhance cell-cell contact (165). In paper III, we show that PDGF-D induces complexes between NRP1 and PDGFRβ, both in *cis* and in *trans*, which further extends the possibilities of modulation of PDGF-D signaling.

NRP1 in PDGF signaling

There is an increasing amount of data showing that NRP1 regulation of PDGF signaling appears to be important for vSMC function, and possibly also endothelial-mural cell communication (42, 43, 140-144). As discussed previously, the main PDGF ligand in the vasculature is PDGF-B, and several studies report that NRP1 can modulate PDGF-B-induced migration of vSMCs through tyrosine phosphorylation of the intracellular integrin adaptor protein p130Cas (141, 143, 166). NRP1 has also been reported to participate in PDGF-mediated recruitment and differentiation of MSCs into pericytes (140). Consistently, several studies have shown that impairment of NRP1 signaling disrupted PDGF-B-mediated vSMC migration (43, 142-144). NRP1 implications in vSMC-related diseases also appear to overlap with the indications for PDGFs; NRP1 contributes to neointimal hyperplasia, and promote the synthetic phenotype of vSMCs, through a pathway that is likely PDGF-dependent (144). NRP1 is also upregulated in response to PDGF-B, thus reinforcing NRP1-PDGF signaling (43).

Physical interactions between NRP1 and PDGFRs have been suggested, but the specific mechanisms for modulation of PDGF/PDGFR signaling by NRP1 are uncertain. Co-immunoprecipitation experiments have shown that NRP1 forms complexes with PDGFRα and PDGFRβ in mesenchymal stem cells when stimulated with PDGF-A or PDGF-B, thus

indicating that NRP1 could act as a co-receptor for PDGFs (42). However, there have been contradictory reports on whether PDGF-B can bind to NRP1 (43, 143). These controversies may be explained by the presence of an additional, 250 kDa form of NRP1, which is glycosylated between the "b2" and "c" domains by chondroitin sulfate (CS) or heparan sulfate glycosaminoglycans. The CS-NRP1 enables cell-specific regulation of binding, and is present in vSMCs and certain tumor cells, where it has been shown to promote PDGF signaling. At the same time CS-NRP1 also inhibits VEGF-A binding to VEGFR2 (167).

We have found that PDGF-D is a much more specific ligand for the 130 kDa NRP1 species, than PDGF-B, and that PDGF-D binds NRP1 with about the same affinity as VEGF-A_{165}. This finding, and the role of NRP1 as a co-receptor for PDGF-D are discussed in paper III.

2 AIMS OF THIS THESIS

With the exception of PDGF-D, the PDGFs and PDGFRs have been well studied in different mouse models with genetic alterations. The aim of this thesis is to explore the physiological role of PDGF-D in health and disease, mainly by analyses of the *Pdgfd*$^{-/-}$ mouse strain.

The specific aims include:

- To map the expression of *Pdgfd*, using the LacZ reporter gene in the *Pdgfd* knockout construct (Paper I)
- To investigate the phenotype the *Pdgfd*$^{-/-}$ mice (Paper I)
- To evaluate the role of PDGF-D ablation in a genetic model of cancer (Paper II)
- To characterize the binding of NRP1 to PDGF-D, and the ability of PDGF-D to induce complex formation between NRP1 and PDGFRβ (Paper III)

3 PAPERS AND DISCUSSION

To highlight central findings and rationale, each paper will be summarized and discussed below. Methods and results are described in detail in the respective papers.

3.1 PAPER I – MICE LACKING PLATELET-DERIVED GROWTH FACTOR D DISPLAY A MILD VASCULAR PHENOTYPE

The roles of PDGF-A, -B and -C, and their signaling through PDGFRs have been studied through a large number of reporter gene, transgenic and knockout animal models, which have contributed greatly to the understanding of PDGF biology. However, the role of PDGF-D is still obscure, and prior to this study, there were no reports of reporter genes or knockout mice for PDGF-D.

In a ligand-receptor system such as the PDGF system, the phenotype of the ligands is expected to match that of the receptor, as seen for the *Pdgfa*$^{-/-}$ and *Pdgfc*$^{-/-}$ phenotypes compared to that of *Pdgfra*$^{-/-}$. The phenotypes of PDGF-B and PDGFRβ are very similar, and therefore, the phenotype of *Pdgfd*$^{-/-}$ mice was expected to be milder. However, PDGF-D exerts its functions through PDGFRβ, which has a very distinct role in vSMCs/ pericytes, where it is needed for mural cell recruitment and proliferation during angiogenesis, and maintenance of the vascular homeostasis in the adult. PDGF-D expression has been reported in the vasculature previously (8, 27-29, 31), although there have been some inconsistencies in the reports of expressing cell types. PDGF-D has also been implicated in a number of vascular pathologies, and therefore, the vasculature was a candidate site of action.

In this study, we present the *Pdgfd*$^{-/-}$ mouse strain, which is viable, fertile and show no gross abnormalities, in contrast to the other PDGF knockouts. The mouse has a normal life span, which enables future studies of PDGFRβ signaling in the adult settings. The study aim was to map the expression of *Pdgfd* through the *LacZ* reporter gene that was inserted in the targeted allele, and to explore the physiological functions of PDGF-D/ PDGFRβ signaling, with the vasculature as a starting point.

To identify organs of interest for PDGF-D function, we performed a general expression analysis on mRNA level, where adrenal gland, spinal cord, aorta, heart, uterus, cerebellum and lung were identified as high expression organs. These are all highly vascularized organs, and also, the vasculature was pointed out as a common expression site of *Pdgfd* between organs in our histology based reporter gene studies, thus supporting the notion that PDGF-D should have a vascular function. In concordance with previously published studies (27, 29), *Pdgfd* expression was seen predominantly in the endothelial compartment, but also occurred in vSMCs. We also confirmed that the *Pdgfd* expression was more prominent in arteries than in veins and frequently found in foci around vessel bifurcations. Moreover, there appeared to be a spatio-temporal regulation of expression, as it went from a strong, exclusively arterial expression at postnatal day (P4) to become more widely spread in adult vasculature, which could be representative of both endothelial-to-mural cell and mural cell autocrine signaling.

In total, our study confirms that PDGF-D can be expressed in both endothelial cells and vSMCs, and that the expression differs depending on vessel type and stage of life.

The other PDGFRβ ligand, PDGF-B, is secreted by the endothelium in its active form but its expression is low or undetectable in the healthy, dormant endothelium. As PDGFRβ is expressed in vSMCs, a possible function for PDGF-D could be in vascular maintenance or endothelial-to-mural cell signaling, where it would be activated upon need. Therefore, we examined vascular morphology and pericyte coverage. While we found no changes in endothelial appearance, nerve/glial antigen 2 (NG2)-expressing pericytes appeared disorganized and less attached in the cardiac vasculature of *Pdgfd*$^{-/-}$ mice. We verified the downregulation of NG2 also on mRNA level by qPCR, and also found another mural marker, desmin, to be downregulated. To explore whether the deviations in NG2^{+} vasculature had implications on *Pdgfd*$^{-/-}$ heart function, we performed a broad gene-expression analysis, including different immunological, developmental and vascular genes. We found a downregulation of *Gata4* and *Notch1* mRNA, two genes that regulate cardiac development and growth, cell fate, and maintenance of cardiac function in adult tissue, and thus, we continued the search of a cardiac phenotype. Clinical chemistry analysis revealed a modest increase in *Pdgfd*$^{-/-}$ serum concentrations of Ca^{2+}, and we observed a trend towards smaller hearts in the *Pdgfd*$^{-/-}$ animals, compared to wildtype controls. Finally, we also detected a slight, but significant, elevation in both systolic and diastolic blood pressure compared to wildtype controls, suggesting a peripheral vascular or cardiac defect in *Pdgfd*$^{-/-}$ mice.

As the *Pdgfd*$^{-/-}$ animals survive, we did not expect to find any major heart defects. However, the slight increase in blood pressure indicates that something is different in the vasculature or the regulation of blood pressure, and it is tempting to speculate that it is related to the abnormalities seen in cardiac NG2^{+} pericyte morphology and/or serum Ca^{2+}. It could also be connected to cardiac output, mean arterial pressure or peripheral resistance, which also affect the blood pressure blood pressure. It would be interesting to see whether anomalies are present also in other PDGFRβ-expressing cells, such as epithelial cells, of the *Pdgfd*$^{-/-}$ mice, and to assess cardiac output, and other cardiac parameters by echocardiography.

In summary, we present a *Pdgfd* knockout mouse strain with a mild vascular phenotype, thereby completing the PDGF "knockout family". The *Pdgfd*$^{-/-}$ mice will be a great aid in studying the physiological and pathological roles of PDGF-D/PDGFRβ signaling, during both developmental and in adult stages of life. We provide new insights on both expression pattern and function of PDGF-D, suggesting a role in regulation of arterial blood pressure. We also describe a possible role for PDGF-D in mural cell physiology and regulation of vascular homeostasis.

3.2 PAPER II – FUNCTIONAL MALIGNANT CELL HETEROGENEITY IN PANCREATIC NEUROENDOCRINE TUMORS REVEALED BY TARGETING OF PDGF-DD

Tumors are heterogeneous, and consist of cancer cells of varying level of differentiation, and varying composition of the stromal compartment. PDGFs are commonly deregulated in

cancer, and do not only act on the tumor cells, but also on PDGFR-expressing stromal cells, such as CAFs, pericytes and vSMCs (reviewed in (112)), where they drive cellular processes such as proliferation, survival and motility, thereby promoting tumor growth and invasion (reviewed in (1)). Pharmacological blockade of PDGF signaling is routinely used for treatment of a diversity of malignancies (105). PDGF-D is upregulated in a number of different tumors, and is known to promote tumor progression through ECM remodeling and deposition. Lately, PDGF-D has also been reported to be involved in EMT and to support of cancer stem cells (130, 133, 136-138).

The aim of this study was to investigate PDGF-D and PDGFRβ signaling in a heterogeneous tumor. For this purpose, we bred the *Pdgfd*$^{-/-}$ animals with the RIP1-TAg2 mouse model of pancreatic neuroendocrine tumors (PanNET). The RIP1-TAg2 mice are engineered to express the oncogenic SV40 T antigen under the rat insulin promotor in the pancreatic islets, leading to hyperproliferation of islets and tumorigenesis. We found that PDGF-D deficiency delayed tumor growth by reducing cell proliferation, and thereby prolonging survival.

RIP1-TAg2 tumor formation has been studied previously in mice lacking the PDGF-B retention motif (*Pdgfb*$^{ret/ret}$) (168). In these mice, pericyte recruitment is severely disturbed in both normal and tumor vasculature, and has also been connected to an increased rate of metastasis. In consistence with our findings in Paper I, the expression of PDGF-D appeared to be mainly vascular in RIP1-TAg2; *Pdgfd*$^{+/-}$ tumors. While vascular insufficiency and low presence of cytokine producing cells are common reasons for tumor growth stagnation, no effects were seen on angiogenesis, pericyte recruitment or immune cell infiltration. Also, the lack of PDGF-D did not affect the invasive nor metastatic properties of RIP1-TAg2 tumors. These findings are in coherence with our findings in Paper I, that the *Pdgfd*$^{-/-}$ mice does not display any gross vascular abnormalities. Moreover, the metastatic properties are tightly connected to vascular integrity, and as the RIP1-TAg2; *Pdgfd*$^{-/-}$ tumor vessel morphology was unaltered, the lack of alterations in metastatic frequency was not surprising.

To better understand the delayed growth in PDGF-D deficient tumors, we did an expression profiling by qPCR that showed an upregulation of *Pdgfb* in RIP1-TAg2; *Pdgfd*$^{-/-}$ mice, compared to RIP1-TAg2; *Pdgfd*$^{+/+}$ controls. This upregulation was likely aimed to make up for the lack PDGF-D, but clearly not enough to compensate for the reduced tumor growth, thus indicating a special role for PDGF-D in tumor development. Therefore, we explored the possibility of PDGF-D-responsive cells outside of the vascular compartment. By co-stainings of insulin and PDGFRβ, in RIP1-TAg2; *Pdgfd*$^{+/+}$ tumors, and fluorescence-activated cell sorting (FACS) analysis, we were able to identify a small subset of non-vascular tumor cells that were strongly expressing PDGFRβ, and thus responsive to PDGF-D. Consistently, a rare PDGFRβ^{+} subset of cells was also present in the malignant βTC3 cell line, derived from RIP1-TAg2 tumors. This was a novel and highly interesting finding, as PDGFRβ expression has been thought to be restricted to mural cells in the RIP1-TAg2 model (169). Interestingly, the βTC3 cells increased proliferation and formed a larger number of tumor spheroids upon treatment with PDGF-D, compared to PDGF-B, indicating a mechanistic difference between

PDGF-D and PDGF-B signaling. It is possible that the newly discovered PDGF-D co-receptor, NRP1, is involved in signaling modulation through altering, enhancing or prolonging signaling response. The function of NRP1 in modulation of PDGF-D signaling is addressed in Paper III.

To investigate the tumorigenic properties of PDGFRβ$^+$ βTC3 cells, FACS sorted cells that were either PDGFRβ$^+$ or PDGFRβ$^-$ were subcutaneously transplanted into immunodeficient mice, and yielded tumors that were histologically indistinguishable from each other. Another FACS analysis showed that tumors formed from PDGFRβ– cells had the same proportion of PDGFRβ expression as tumors formed from PDGFRβ$^+$ cells, thus indicating an inter-conversion between these two subsets of cells. We also verified that a subpopulation of PDGFRβ$^+$ malignant cells is present also in human PanNET.

Taken together, we show that PDGF-D may contribute to PanNET tumor growth through angiocrine signaling, reinforcing functional malignant cell heterogeneity by stimulation of a rare PDGFRβ$^+$ subset of tumor cells, which then engage in paracrine crosstalk with neighboring PDGFRβ$^-$ cells to drive tumor progression. We also show that PDGFRβ$^-$ tumor cells can switch into a PDGFRβ$^+$ phenotype, to maintain a constant level of PDGF-D-responsive cells, thereby securing local access to tumorigenic signals. It would be interesting to further characterize NRP1 expression and its putative role in regulation of PDGF-D signaling in the progression of PanNET. Lastly, the notion that PDGFRβ$^+$ malignant PanNET cells exist will likely impact the treatment regimens used for this type of cancer in the clinic. Sunitinib and other PDGFRβ-targeting drugs might not only affect the vascular function, but also this rare population of highly tumorigenic cells, thus positioning these drugs as even more potent treatment options for PanNET than previously thought.

3.3 PAPER III – NEUROPILIN 1 IS A CO-RECEPTOR FOR PLATELET-DERIVED GROWTH FACTOR (PDGF)-D/PDGF RECEPTOR (PDGFR)β SIGNALING

The need for PDGF-B signaling through PDGFRβ signaling in angiogenesis and vascular homeostasis is well documented. However, the role of PDGF-D is less clear. The existence of PDGF-D as a PDGFRβ specific ligand, together with its need for extracellular activation, and the pathological implications from overexpression studies confirm that, once activated, PDGF-D is very potent in altering ECM homeostasis and other processes. Indeed, PDGF-D can induce responses separate from those induced by PDGF-B. As shown in Paper II, PDGF-D, but not PDGF-B, induces signaling in rare PDGFRβ-expressing tumor cells, and contributes to tumor progression in PanNET in a manner that cannot be explained by differential activation. This could, however, be explained by modulation of signaling by a co-receptor.

There is a growing body of literature showing that NRP1 can modulate the signaling of both PDGFRs (42, 43, 140, 142, 143), but it does not bind to PDGFRβ directly. Also, the affinity of PDGF-B binding to non-glycosylated NRP1 appears to be low (42, 143). Hence, this opens up for an additional, co-receptor-modulated role of PDGF-D as the other ligand for PDGFRβ.

In this study, we discovered that PDGF-D shares some structural features with NRP1 binding VEGF ligands. In similarity with the NRP1-binding VEGF-A isoforms, VEGF-C, and VEGF-D, the PDGF-D growth factor domain has a conserved cysteine knot structure, accompanied by a C-terminal positively charged arginine residue, which in the VEGF ligands is crucial for NRP1 binding. However, these structural features are not seen in PDGF-B.

We show that PDGF-D specifically binds to NRP1 and induces PDGFRβ/NRP1 complex formation in cells, both in *cis*- and in *trans*-conformation, and that binding leads to phosphorylation of PDGFRβ, thus inducing downstream signaling. We also confirm that NRP1 binding of PDGF-B is only limited. Moreover, upon PDGF-D-stimulation of cultured cells, PDGF-D, NRP1 and PDGFRβ were co-clustered and internalized together, suggesting that NRP1 alters the trafficking of PDGFRβ. A similar mechanism has previously been shown for NRP1 in modulation of VEGF-signaling (170).

To our knowledge, this is the first study to assess the role of the novel PDGFs (PDGF-C or PDGF-D) as targets for NRP1 modulation. It makes sense that PDGF-D would be the preferred PDGFRβ ligand for NRP modulation, considering that PDGF-D expression and activation are not coupled. With these new insights into the co-receptor function of NRP1 for PDGF-D/PDGFRβ signaling, our study provides a mechanistic basis for differential signaling between PDGF-D and PDGF-B. It also adds complexity to the current hypotheses on NRP1 modulation of PDGF signaling and might even challenge some conclusions drawn from the previous reports.

Altogether, these findings will lead to a better understanding of the role(s) of PDGF-D, and thereby to improved development of tailored therapeutics for conditions where vascular function is disrupted, such as atherosclerosis and cancer.

4 FUTURE PERSPECTIVES

In the work included in this thesis, we present the *Pdgfd*$^{-/-}$ mouse strain, and thereby the "PDGF family of knockouts" is finally complete. The mild phenotype of the *Pdgfd*$^{-/-}$ mouse strain (described in Paper I) was not surprising to us, given the similarity between the PDGF-B and PDGFRβ phenotypes, indicating a certain amount of redundancy between PDGF-D and PDGF-B during physiological conditions. However, PDGF-B and PDGF-D signaling mechanisms differ in that PDGF-B is secreted in its active form, and retained close to the cell of origin through ECM binding, while PDGF-D is released in an inactive, diffusible form. Thus, PDGF-D expression and activation are uncoupled, and require the presence of additional activating factors, indicating a potent inducible function of PDGF-D, which might be important under specific conditions. Moreover, the identification of NRP1 as a co-receptor for PDGF-D, and its ability to form signaling complexes with PDGFRβ also in *trans* indicates that NRP1 does not only modulate signaling in the PDGFRβ -expressing cell, but also enables and potentiates cell-cell communication via direct adhesion to neighbouring NRP1-expressing cells. In my mind, this implies that a "main" function of PDGF-D is yet to be found.

In the vasculature, NRP1 is expressed by endothelial cells, and PDGFRβ by mural cells, thus the proposed signaling in *trans* is highly relevant for the communication between these two cell types. Interestingly, PDGF-D is commonly expressed around vascular bifurcations, and we have hypothesized that it is involved in regulation of local blood flow. Could it be that endothelial NRP1 acts in complex with mural PDGFRβ to regulate vascular contraction? This could be studied through an aortic ring assay, with and without PDGF-D stimulation. Another idea is that extra stability is needed in these areas due to turbulent blood flow, and that this is mediated through the NRP1-PDGF-D-PDGFRβ *trans* complexes.

It is also possible that PDGF-D has a role in angiocrine signaling with effects on cells in the tissues surrounding the vessels that could be somewhat similar to the mechanism described in Paper II. In this study, we present evidence that vascular PDGF-D reinforces the proliferative paracrine signaling of a rare population of PDGFRβ-expressing tumor cells to neighbouring tumor cells. Thus, the main site of action for PDGF-D might not be the vasculature, but some other tissue at some distance from the vessel. Interestingly, PDGFRβ expression has been reported in non-vascular progenitor cell populations and PDGF-D has been connected to stem cell signaling also in non-malignant conditions (60, 73, 74). Our reporter gene study indicated prominent expression of PDGF-D in several different epithelial tissues. Whereas epithelial cells are known to produce the PDGFRα-specific ligand PDGF-A (reviewed in (2)), the role of PDGFRβ in epithelial cells is not clear. However, the strong reporter gene expression of the PDGFRβ-specific PDGF-D is indicative of a role also for PDGFRβ signaling in the epithelium, possibly in a long-range paracrine manner similar to angiocrine signaling.

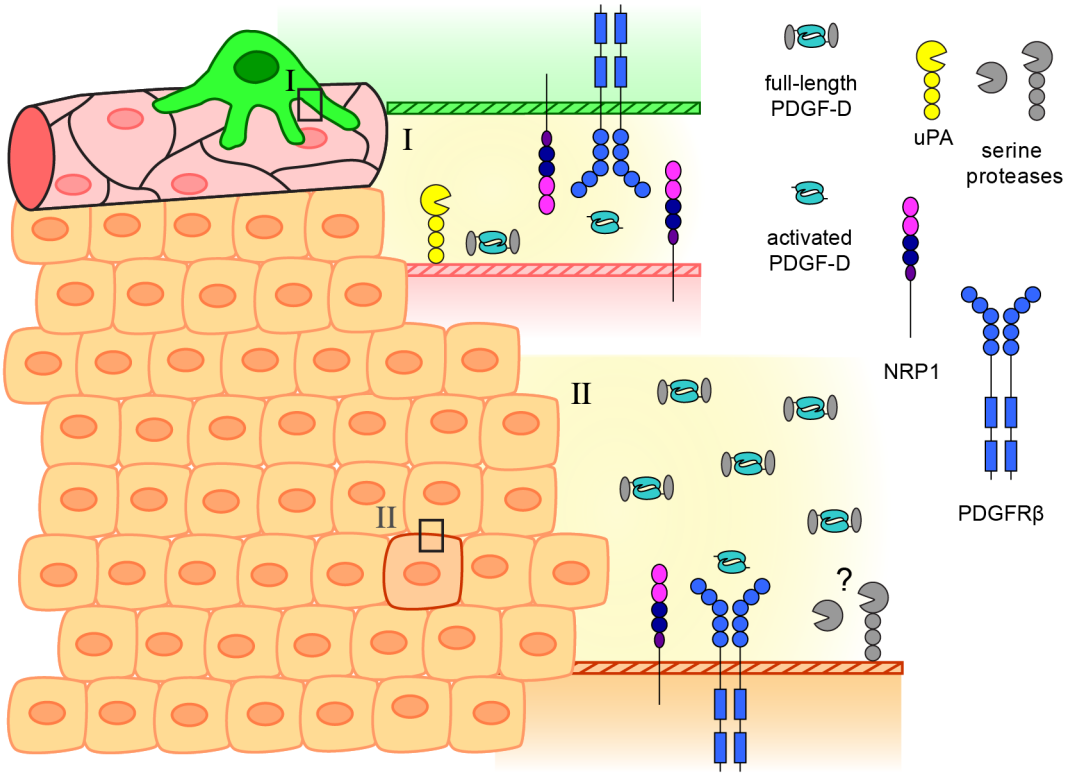

Figure 3. Possible physiological signaling modes for PDGF-D. PDGF-D is expressed by endothelial cells and pericytes, and activated by uPA, matriptase or possibly other unknown serine proteases. uPA is expressed by endothelial cells. (I) Short range paracrine or autocrine signaling in the vasculature. Pericytes express PDGFRβ, and PDGF-D can either bind its receptor directly or engage the co-receptor NRP1 to form a complex. Complex formation can occur both in *cis* and in *trans*. (II) Angiocrine signaling, from the vasculature to cells in the surrounding tissue. The latent PDGF-D is diffusible and therefore not retained in the vasculature. Hypothetically, rare cells in the tissue express PDGFRβ, and the same cells, or surrounding cells express activating serine proteases and the co-receptor NRP1. Thus, NRP1-modulation of PDGF-D/PDGFRβ signaling in *cis* or *trans* could occur.

Now, the next step is to challenge the *Pdgfd*$^{-/-}$ mice, to see if it has any phenotypic deviations compared to the wildtype mice. We have several projects involving challenges going on in the lab, and Paper II is derived from one of them. Another condition where PDGF-D is strongly implicated is atherosclerosis, and the SNPs in PDGF-D associated with coronary artery disease indicate that dysregulation of PDGF-D aggravates the disease. In an ongoing study, we have crossed the *Pdgfd*$^{-/-}$ mice with the athero-prone *ApoE*$^{-/-}$ mice. It will be very interesting to analyze the aortas from these mice to see if the plaques are differently constituted, and what cell populations that are altered.

In any case, it is possible that NRP1 holds the key to the function of PDGF-D, and this will be considered in future studies.

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